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THE UNIVERSITY OF MICHIGAN

SCHOOL OF DENTISTRY
DEPARTMENT OF ORAL SURGERY

Annual Progress Report

Promotion of Primary and Rapid Osteogenesis in Healing Maxillofacial Bone Injuries

JAMES R. HAYWARD
GERALD H. BONNETTE
ROBERT A. BRUCE
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THE UNIVERSITY OF MICHIGAN

SCHOOL OF DENTISTRY
Department of Oral Surgery

Annual Progress Report

PROMOTION OF PRIMARY AND RAPID OSTEOGENESIS IN HEALING
MAXILLOFACIAL BONE INJURIES

James R. Hayward
Gerald H. Bonnette
Robert A. Bruce
Gilbert S. Small

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PART I

ANALYSIS OF CHONDROGENIC INFLUENCES RELATING TO DELAYED BONE REPAIR

INTRODUCTION

Gunshot wounds, especially modern high velocity missiles, produce comminuted maxillofacial bone destruction which heals slowly with an associated extensive cartilage production. The healing complications include prolonged hospitalization, protracted convalescence, and significant permanent disability.

The ultimate goal in treating extensive injury of the maxillofacial skeleton is to promote primary bone repair. The repair should progress directly to bone without a stage of cartilage formation. Oxygen supply and tissue consumption of oxygen may determine the type and rate of osteogenesis. If the relationship between cartilage production (delayed healing) and oxygen deficiency can be evaluated under experimental controls with an injury model in animals, methods could be developed to increase local tissue oxygen supply and increase the rate of osteogenesis since chondrogenesis commonly associated with delayed and inadequate healing would be eliminated.

Healing of maxillofacial bone injuries is a unique process compared to bones in other anatomical sites. Generally, bones of the maxillofacial complex develop through an intramembraneous rather than an endochondral ossification. Also the vascular supply to the head and neck is more extensive and complex as compared to other bones. Factors such as teeth within the fracture area, oral wound contamination, and masticatory impairment with nutritional deficiency, all present unique challenges to the repair process.

Treatment of comminuted maxillofacial bone injuries favors the use of close reduction techniques which maintain bony fragment attachment to the associated periosteum. This insures the viability and regeneration potential of the comminuted fragments. The hematoma which is formed, organizes and eventually forms cartilage which must be removed through a process of endochondral ossification before bone is ultimately formed.¹ It has been noted, however, that non-comminuted and less extensive bone wounds heal through a process of intramembraneous bone formation, directly to fibrous bone without an initial cartilage phase.²

The literature has described cartilage production during healing to be related to a number of variables such as disruption of adjacent periosteum, size of bony defects, mobility of fracture fragments, species and age of experimental animals and disruption of vascular supply. A common denominator appears to relate cartilage to diminished vascular supply and tissue hypoxia.^{3,4}

Experimental studies of regenerating bony tissue have utilized compression to decrease the vascular supply and have shown a decrease in hemoglobin content, a decrease in oxygen consumption, a decrease in tissue lactic acid, an inactiva-

tion of the cytochrome system, and an increase in local mucopolysaccharide production.⁵ Tissue culture experiments have related high carbon dioxide concentration to the production of chondroblasts from mesenchymal tissue.^{6,7}

Histochemical studies have identified Adenosine Triphosphate (ATP) and Desoxyribose Nucleic Acid (DNA) in both cartilage and bone. The presence of a functioning Krebs cycle has also been identified in various types of cartilage and has been shown to be dependent on aerobic conditions.⁸ The cytochrome oxidase system has been identified in cartilage and is known to catalyze oxygen consumption resulting in the production of ATP.

Since ATP production is affected by oxygen supply and utilization, tissue ATP levels may represent a quantitative measurement of oxygen utilization of regenerating osseous tissue. Quantitative DNA levels reflect the degree of cellularity in a given tissue sample since the amount of nuclear DNA tends to be constant. Thus a ratio of ATP to DNA is a precise measure of the levels of ATP per cellular unit.

This study was designed to compare histologically healing in standardized bone defects with and without loss of bony continuity and to utilize this model to quantitate ATP and DNA levels in regenerating undifferentiated tissue before additional healing resulted in cartilage or bone.

MATERIALS AND METHODS

HISTOLOGICAL ANALYSIS

Twenty New Zealand adult white rabbits were used to evaluate the effect of a fracture on cartilage production. A standardized 3 mm defect was made both in the mid-portion of the left tibia and the left body of the mandible utilizing a trephine bone bur (Figure 1). Identical defects were also made in the right tibia and right mandible and in addition, a cut was made through the remaining bone with a No. 1 round dental bur (Figure 2). In the mandible, the cut was made so as to produce a so-called "favorable fracture" which created mobility of the fragments without gross displacement. The fracture through the tibia was positioned so that the fibula initially acted as splint to prevent gross displacement of the fragments. However, with function, the fibula was unable to maintain alignment of the fractured tibia and the fragments were grossly displaced. Efforts at obtaining fracture reduction with wires or plaster casts were unsuccessful.

The animals were sacrificed 7, 9, 12, and 15 days after surgery. The surgical areas were processed and stained with Masson's and Alcian Blue for histologic evaluation.

Since we were interested in obtaining standard amounts of tissue for biochemical analyses, we were not able to use the tibia for analysis since there was such gross displacement of the fragments.

BIOCHEMICAL ANALYSIS

Sixteen adult New Zealand White rabbits were used to analyze the healing tissue for ATP and DNA. With the use of a 3 mm trephine bur, a defect was made in the left mandible and a defect with a fracture was made in the same position in the right mandible. Since a previous histologic study has shown no apparent difference in the fracture and the defect at 7 days of healing, it was decided to compare the tissue samples biochemically from the fracture and the defect at this time. The 16 animals were sacrificed one week following surgery and the healing tissue from the right and left mandible were removed in the form of a plug by the use of a hand-rotated trephine bur. The tissue was homogenized and one-half of the homogenate analyzed for ATP utilizing the method of Strecher and Totter.⁹ The method is essentially a luminescence assay of the ATP utilizing firefly extract. The tissues were homogenized in one milliliter of distilled water in a Kontes-type tissue homogenizer cooled in ice water. The homogenate was boiled for ten minutes and 0.2 ml of the sample was added to 0.2 ml of firefly extract reagent and 0.6 ml of distilled water. The amount of luminescence was read at 15 sec in a Farrand Photofluorom-

eter and the reading converted to micrograms of ATP per 100 mg wet tissue by comparing it to a standard curve.

The remaining half of the homogenate was analyzed for DNA using the method of Munro and Fleck.¹⁰ The method involves a spectrophotometric analysis for DNA utilizing color change of indole. Two ml of the original homogenate was added to 1.2 ml of distilled water and 0.6 ml of 0.6 N perchloric acid. The sample was cooled for ten minutes and centrifuged for 10 min. The supernatant was discarded and the precipitate washed with 0.2 N perchloric acid. One ml of 0.3 N KOH was added to the precipitate and incubated at 37° for 60 min. The sample was cooled for 10 min, again washed with 1 ml of 0.6 n perchloric acid, centrifuged for 10 min, and finally washed twice with 1 ml of 0.2 n perchloric acid. The sample was diluted with 2 ml of distilled water, and to 2 ml of the sample, 1 ml of 0.04% indole and 1 ml concentrated hydrochloric acid was added. The sample was boiled for 10 min, cooled, and extracted three times with purified chloroform. The samples were read at 487 mμ in a Beckman DU spectrophotometer and the values were converted to mg of DNA per mg wet tissues by comparing to a standard curve.

RESULTS

HISTOLOGIC

Histologic results showed mesenchymal tissue to be present in the defects after 7 days of healing (Figures 3,4). Various gradations of healing were noted through 9 and 12 days and in general the defects were filled with fibrous bone after 15 days of healing (Figures 5,6). No cartilage was noted in the healing defects, either in the mandible or tibia, and bone formed apparently through an intra-membranous type formation. There was no noticeable difference in healing between the fractures and the defects after 7 days, but in the 9-, 12-, and 15-day specimens, cartilage was abundant in the fractures (Figures 7,8). Healing progressed in a typical endochondral type of ossification in which cartilage was replaced by bone. Fifteen days after surgery, cartilage had not been completely replaced. The histologic results were relatively consistent and there were no apparent healing differences noted between the mandible and tibia.

BIOCHEMICAL

There was more ATP present in the bony defects than in the fractures, but less DNA in the defects than in the fractures (Table I). A student Fisher t-test of the results showed significance to be at the 0.02 level. A ratio was

TABLE I

	DEFECT	FRACTURE	COMPARISON
ATP	0.95 \pm .16	0.78 \pm .18	P .02
DNA	6.5 \pm 2.5	9.7 \pm 4.4	P .02
ATP/ DNA	0.17 \pm .09	0.10 \pm 0.05	P .02

Values \pm standard deviation
 ATP values in $\mu\text{g}/100\text{mg}$ wet wt.
 DNA values in $\mu\text{g}/\text{mg}$ wet wt.
 N = 16

made of the ATP to the DNA in each sample and the significant differences between the fractures and the defects were again noted at the 0.02 level.

DISCUSSION

The results suggest that cartilage is produced when there is a loss of continuity of the bone as in a fracture. There appears to be presumptive evidence from various other studies to indicate that cartilage is related to a decreased blood supply. However, it should be noted that there have been several studies of healing of bony defects in long bones as well as the mandible in which the major arterial supply has been removed and cartilage has not been produced unless there was an associated fracture. Perhaps this can be explained by collateral circulation.

Our results suggest that local tissue oxygen supply or utilization as measured by ATP production is decreased early in the healing process and may account for development of cartilage in the fracture site. It is interesting to note the decreased cellularity in the healing defects as compared to the fracture as evidenced by DNA levels. One can only speculate that perhaps more cells are needed in the relative hypoxic environment since efficient energy production is curtailed.

Since statistically significant quantitative differences in tissue oxygenation have been noted between the mesenchymal tissue which progresses to cartilage and the bone forming mesenchymal tissue, our future studies will be enlarged to evaluate methods of early oxygenation of healing osseous tissue. These studies will lead to methods to promote primary osteogenesis and, if the cartilagenous stages are by-passed, eliminate prolonged and delayed healing of maxillofacial osseous wounds.

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Figure 1. Three mm trephine defect in the mandible of a New Zealand White rabbit. Tissue samples were removed from this defect 7 days after surgery.



Figure 2. A combination of a trephine defect and a fracture through the defect. Note the fracture line is positioned to allow mobility without gross displacement of the bony fragments.



Figure 3. Histologic section of the mandibular defect 7 days after surgery. Note the mesenchymal tissue. The same type of tissue was noted in the fracture specimen.



Figure 4. Higher power of specimen in Figure 4.



Figure 5. Histologic section of a defect in the tibia 15 days after surgery. Note the lack of cartilage and the abundance of fibrous bone.



Figure 6. Histologic section of a defect in the mandible 15 days after surgery. Note the fibrous bone and the lack of cartilage.



Figure 7. Histologic section of a fracture through the tibia after 15 days of healing. Note the cartilage on the periosteal surfaces and the displacement of the bony fragments.



Figure 8. Histologic section of a fracture through the mandible. This section demonstrates the abundance of cartilage in the fracture site.

PART II

OSTEOGENIC INDUCTION WITH BONE MARROW

The reasonable hypothesis of this study is that bone formation does occur under a variety of extraskeletal environmental situations and that bone marrow is known to induce bone formation in an extraskeletal environment. From previous work by others,^{15,16,17,28} there appears to be a diffusible substance which can stimulate cells to induce new bone formation in a receptive environment.

A laboratory method to demonstrate bone induction was devised by Goldhaber¹⁵ using a Millipore Chamber as a vehicle and fetal mouse calvarium as a substrate to induce bone formation outside the chamber. On this basis, it was thought that there is a diffusible substance which stimulates mesenchymal cells to differentiate to osteoblasts and to form bone. This work was corroborated by Heiple and others²⁸ and has been refined in addition by using the electron microscope to try and identify materials which pass from the interior of the chamber through the filter to induce cellular differentiation to form bone outside the chamber. The osteogenic potential of bone marrow is considered low, but has been demonstrated in numerous laboratory models. Burwell's study^{18,19} used ilium from rats and he attenuated this bone to a variety of forms used in bone graft methods. To this, he added host red marrow. He used the attenuated ilium without bone marrow as controls. These were placed in a host rat. No new bone was seen in the controls, but variable amounts of new bone were seen in the composite homograft-autograft, demonstrating the osteogenic potential of bone marrow in a suitable environment.

This study used the Millipore Chamber isolation. The chamber was made up of cellulose acetate filters, which are 150 μ thick with a pore size of 0.45 μ , a lucite ring, and adhesive (Figure 1). These materials were sterilized with ethylene oxide. The chamber was constructed by gluing a filter to one surface of the ring. Marrow from a donor mouse then was placed on the inner surface of the filter and the chamber was completed by gluing a second filter to the outer surface of the ring.

The first experiment used 20 Spartan mice as recipients. The bone marrow was obtained from the femurs of Ajax mice. Both donor and recipient animals were anesthetized with appropriate doses of sodium nembutal. The femur of one Ajax mouse was dissected free and the condylar heads removed, leaving only the shaft of the femur. One end of the shaft was grasped with a hemostat and the shaft was compressed with cotton pliers to extrude the bone marrow at the other end (Figure 2). Approximately 1 ml of bone marrow was obtained. This was placed on the filter which was kept moist with a sterile physiologic tissue culture solution to prevent dehydration of the marrow. The chamber was then sealed. A mid-dorsal incision was made through the skin of the recipient mouse and then undermined widely to allow room for the chamber. The chamber was placed in the pocket with the filter surface supporting the marrow lying on muscle (Figure 3). The skin was closed with sutures. The chambers were retained in place for 8 weeks.

At the end of 8 weeks, the recipients were sacrificed by cervical dislocation and the implanted chambers were recovered. They were placed in formalin fixation and after proper dehydration and decalcification, were covered with a thin layer of paraffin. The lucite rings were removed with the least amount of distortion by use of a special punch designed for this purpose. The specimens then were placed in paraffin blocks and sectioned at 10 μ . These sections were stained with Lisson's Alcian Blue.

A second series similarly prepared, included 5 animals in which calvaria was placed and 2 in which no material was placed in the chambers for further controls. These two series of experiments were designed to understand the particular technical problems associated with the procedure and also to establish controls.

Study of the marrow sections with a light microscope was disappointing. Most of the original specimens did not show any viable bone marrow, much less new bone formation. It was of concern that the amount of bone marrow from one femur was scanty in the original series. Therefore, in three other chambers, the marrow of 3 femurs was used. These did show bone formation inside the chamber and did induce bone to form outside the chamber (Figures 4, 5, 6). The chamber in which bone was seen growing was interpreted by two separate investigators in oral histology for their evaluation. The bone was noted to stain characteristically with Lisson's stain and showed lamellated character with viable osteocytes. Directly across the filter barrier from this bone, induced new bone was seen on the outside of the chamber, which showed identical histologic characteristics. This new bone was of smaller quantity than that which was noted inside the chamber. Also noted in the chamber and at a site distant from the area where induced bone was seen, there was an area of new cartilage formation (Figures 7, 8). Cartilage was not in contact with the filter paper but was peripherally located in bone and seemed to be in continuity to this. The presence of cartilage was somewhat puzzling. It could represent cartilage from the condylar head of the femur which was inadvertently placed in the chamber. It also may represent a more primitive mesenchymal response of the reticulum cell of bone marrow, which proceeded to form cartilage under a probable lowered oxygen tension environment.

In order to further study these phenomena, it was felt that special calcium stains were necessary to determine histologically if the observations truly indicated bone calcification, since the original material had been decalcified. Four Millipore Chambers were implanted with bone marrow from 3 femurs and allowed to grow in host mice. These were recovered after 8 weeks and were stained by Von Kossa's method. These slides, as noted in Figures 12 and 13, showed that the material inside the chamber was indeed calcified bone by the precipitation of silver salt in this stain. It was also noted in this particular specimen, that there was no induced bone present after 8 weeks (Figures 10, 11).

In an effort to try and understand the sequence in time evolvement of bone maturation and possible induction, 6 other preparations of Millipore Chambers in recipient mice were completed. It was planned to sacrifice these after 2 weeks. Two of these mice promptly died. Material from the other four is shown and is stained with Von Kossa's calcium stain, hematoxylin and eosin, and Lisson's Alcian Blue (Figures 16-22). Comparative stains in these figures show calcification has occurred inside the chamber in 2 weeks with active tissue proliferation about the marrow. The contact of new bone with the filter surface was close. This was also noted in previous material in which bone formation occurred.

Von Kossa's stain, done concomitantly in this material, confirmed active calcification. The marrow at 2 weeks, took on a myxomatous character. Most of the cells had a fibroblastic appearance. Some of the cells showed eosin staining fibers associated with the cellular elements which are interpreted as collagen fibers and are precursors to active mineralization of bone (Figures 22, 23). It was noted in these slides also, that the cellular elements opposite the calcified areas showed some degree of maturity as evidenced by plumping of these cells.

In addition to this 2-week material, 2 mouse preparations were allowed to remain alive for 8 weeks. The chambers from these animals were recovered and stained for alkaline phosphatase activity (Figure 14, 15). As the photomicrographs indicate, alkaline phosphatase activity was very heavy around the calcified areas and the cellular area opposite this calcified area showed maturation and a more heavy precipitate of alkaline phosphatase intracellularly. Although there was no bone induction outside the chamber, the alkaline phosphatase activity did indicate that bone formation perhaps was starting there. At the present time, material is being prepared to assess alkaline phosphatase level at 2 weeks of activity.

After the original experimental series of this research project was completed, the investigators were quite disappointed in the lack of positive indications of bone induction. In reviewing the problem, it was felt that this was based on three causes: (1) that of preventing secondary infection, (2) the environment of the small amount of bone marrow present in the rather large Millipore Chamber, and (3) the inherent low osteogenic capacity of bone marrow.

The first and second portions of this evaluation have been corrected by a stricter attention to sterile technique, including antibiotic coverage, in the diet of these animals. In addition, as noted, the quantity of marrow has been increased in the chambers with the possible formation of an organ colony rather than a cellular colony. In addition, material of smaller size than the Millipore Chamber from the Millipore Corporation is being investigated. At present, further study with tubes of Millipore material, 2 mm in diameter, is under consideration. Nylon reinforced cellulose acetate material of the same pore size will be obtained from the Gelman Manufacturing Company in Ann Arbor,

and this material will be cut to size so that a smaller envelope can be constructed. Neither of these two isolation carriers will be using a plastic ring. It is hoped that increasing the volume of marrow and housing it in a smaller container will allow for more cellular activity.

It appears that marrow can display its osteogenic potential in the first two or three weeks, but then may degenerate. It is hoped that by decreasing the space in which the marrow is housed, it will eliminate a source of decreased oxygen tension and allow better organization of bone marrow potential.

The results so far may be summarized as follows: (1) Bone marrow in a Millipore Chamber can form mature bone. (2) Such bone can be induced outside the Millipore Chamber. (3) The ability of bone marrow to accomplish this under these investigative parameters so far has been low. (4) Bone formation does occur in 2 weeks. (5) Complete bone formation is necessary inside the chamber before bone induction will occur outside the chamber. (6) There is an approximate 6-week time lapse before bone induction will occur.

Further investigative studies in this laboratory are continuing. Presently, further controls are being completed to qualify the experimental bases. These include further empty chambers, chambers filled with muscle, and chambers filled with liver. In addition to the already mentioned discontinuance of the Millipore Chamber itself, and using tubes of Millipore filter and envelopes of cellulose acetate material, chambers are being filled with diced ilium from mice which do adequately fill the Millipore chamber. It is felt that this material, which does represent a particulate graft, may have promise as a more efficient working model for further study of the induction phenomenon.

With the establishment of a working model and that calcification of bone starts at least as early as 2 weeks, it is hoped that by use of biochemical analysis and substitute materials, a base line will be established by which bone induction can be better understood and may allow for an acceleration of repair processes in jaw fractures.

A portion of the above material was presented at the IADR meeting at Houston, Texas in March 1969.

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Figure 1. Millipore assembly apparatus

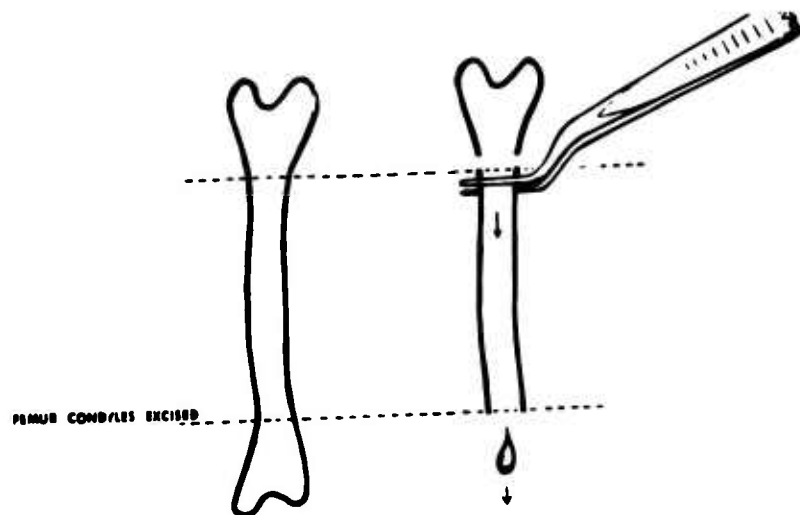


Figure 2. Diagrammatic sketch showing the extrusion of bone marrow from mouse femur.

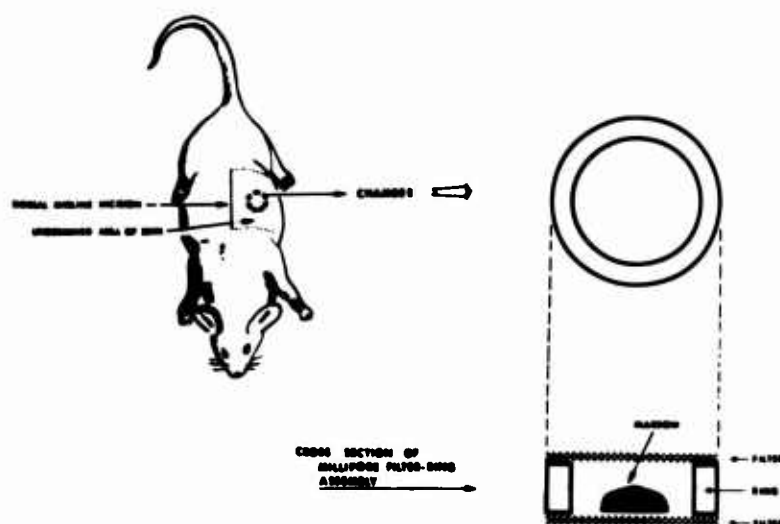


Figure 3. Diagrammatic sketch showing placement of marrow in the chamber and placement in the host mouse.



Figure 4. Punch constructed for removal of plastic ring.



Figure 5. Lisson's Alcian Blue stain showing bone formation, filter paper and induced bone outside the chamber. 100X.

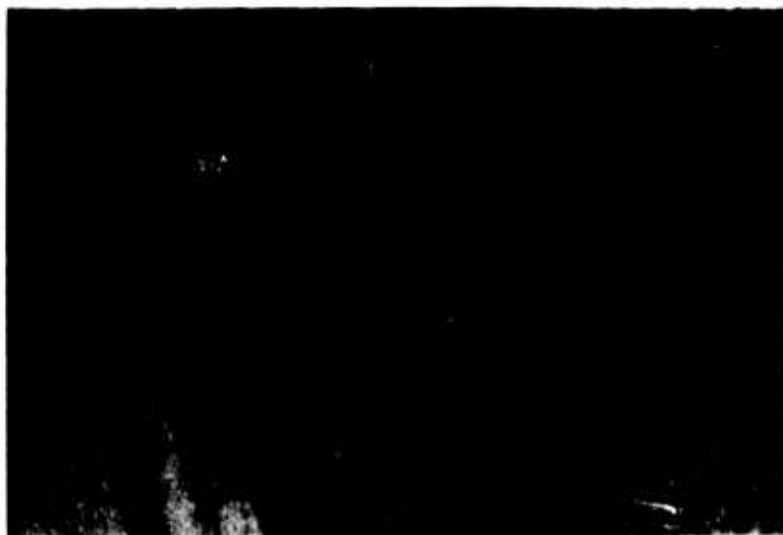


Figure 6. Higher power of bone formation. 250X.



Figure 7. Higher power of induced bone. 250X.



Figure 8. Cartilage formation within the chamber associated with bone formation. 100X.

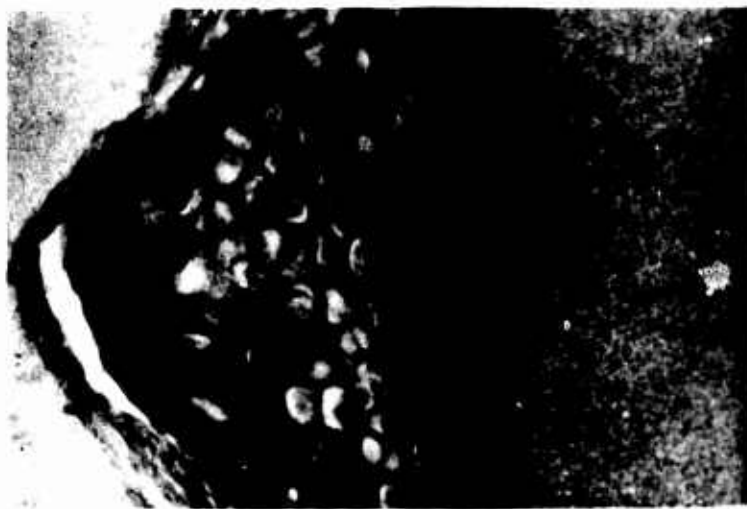


Figure 9. Higher power of cartilage. 400X.



Figure 10. Eight-week specimen, Lisson's Alcian Blue showing calcification inside of chamber. 100X.



Figure 11. Same, higher power. Calcification appears inside pore network. Artifactual detachment from filter surface. Cells outside of chamber appear to be lining up in orderly fashion suggestive of maturation toward osteogenesis. 250X.



Figure 12. Von Kossa's stain. Block precipitation of calcification inside of chamber. 8 weeks. 100X.

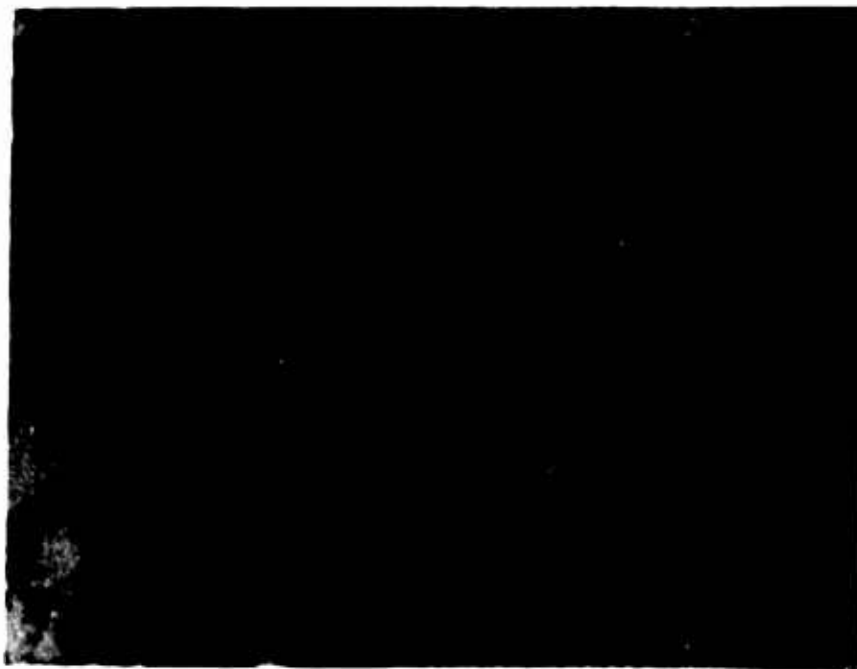


Figure 13. Higher power Von Kossa's stain. Calcification within filter can be seen. 250X.



Figure 14. Gomori's method for alkaline phosphatase, showing intense activity inside of chamber and immediately outside of chamber. 8 weeks. 100X.

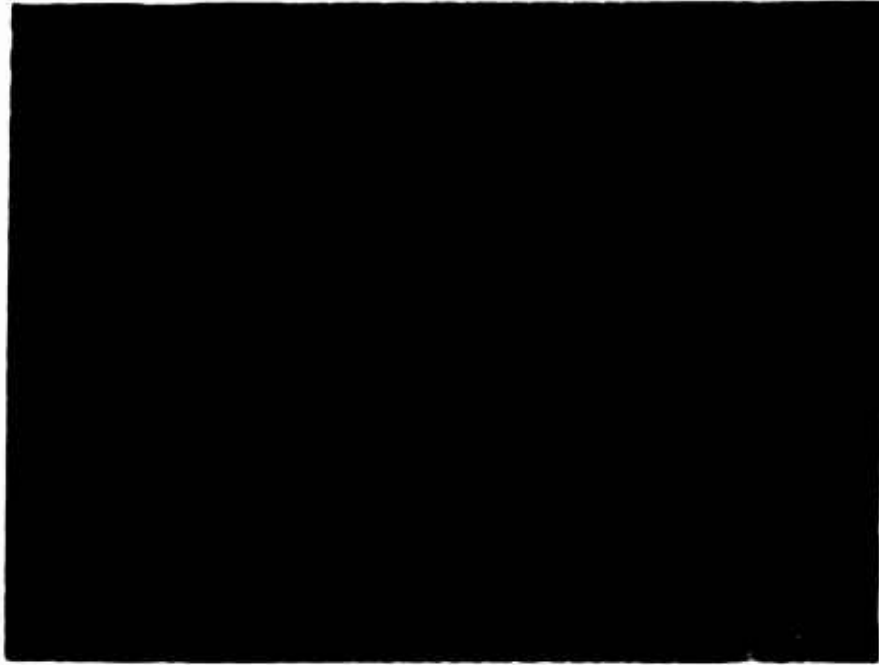


Figure 15. Higher power of same. 250X.



Figure 16. Two-week old specimen with Lisson's Alcian Blue, showing organization of bone marrow with osteogenesis proceeding. 100X.



Figure 17. Higher power. Showing cellular activity towards bone mation.
Some fibroblastic appearing elements appear to be lining up on sur e of
filter. 250X.



Figure 18. Von Kossa's stain to show presence of calcification. 100X.



Figure 19. Higher power of same. 250X.



Figure 20. H and E 2-week specimen, different area showing osteogenesis and myxomatous nature of organizing bone marrow. Cellular elements appear to be lining up on surface of filter. 100X.



Figure 21. Higher power of same. 250X.



Figure 22. H and E 2-week specimen showing characteristics of organizing bone marrow with cells lining up on filter, fibroblastic character of cells. A lymphocyte is noted on left surface. 250X.

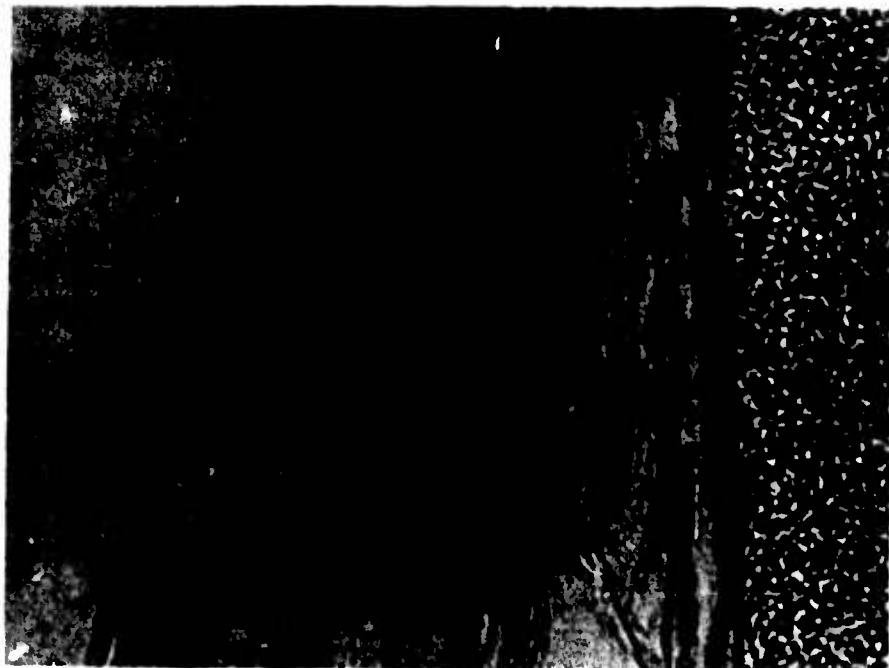


Figure 23. Higher power of same field. Collagen fiber is more apparent.
500X.

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13. ABSTRACT <p>The study of bone marrow as an osteogenic induction agent in mice is progressing. It has been found that in Millipore Chambers, bone marrow can form bone and induce bone outside of the chamber. This potential is low. In addition, it has been found that bone formation occurs as early as two weeks within the chamber, and that bone induction outside the chamber does not occur simultaneously, but is delayed for a period of about six weeks. Further studies are underway to establish a more efficient model to study bone marrow activity in a millipore environment.</p> <p>A study was designed to compare healing histologically in standardized bone defects with and without mobility and to quantitate ATP and DNA in standard healing bone defects and fractures. Histologic results showed cartilage to be related to a lack of bony continuity of the mandible. ATP levels were less in the fractures and DNA levels were less in the defects. The results suggest that cartilage present in bone fractures is related to local tissue oxidative metabolism. Further studies will be designed to increase oxygen to healing fractures to prevent delayed and nonunion healing.</p>		

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